

Supplementary Information

Supplementary Materials and Methods

Isolation of human primary immature dendritic cells. Human immature dendritic cells (iDC) were generated from CD14⁺ monocytes isolated from freshly heparinized single donor blood. Briefly, CD14⁺ monocytes were prepared by Ficoll (Amersham) gradient centrifugation followed by magnetic-activated cell sorting (MACS) of the resulting peripheral blood mononuclear cell population for CD14⁺ positivity using magnetic bead-coupled CD14 antibodies (Miltenyi). Enriched CD14⁺ monocytes were cultured for 5 days in serum-free media (Cellgro, Cellgenix) supplemented with GM-CSF (500 U ml⁻¹) and IL-4 (50 U ml⁻¹) (Miltenyi) to obtain CD14/CD80/CD83/CD86/low iDCs. The protocol was approved by the Ethics Committee of the University Freiburg Medical Center, Freiburg, Germany, and conforms to the principles set out in the WMA Declaration of Helsinki.

Flow cytometry. For intracellular IL-8 detection, cells were processed as described using appropriate fluorochrome-labelled antibodies [1]. Successful transfection of the HA-TLR4 constructs was monitored by staining with rat anti-HA primary antibody (3F10, Roche) prior to detection with a FITC-coupled anti-rat secondary antibody (Dako Z0109). IL-8 was analyzed after gating on HA-positive cells using a specific mouse anti-IL-8 primary antibody (G265-8, BD Biosciences) and a Cy5-coupled anti-mouse detection antibody (F0261, Dako).

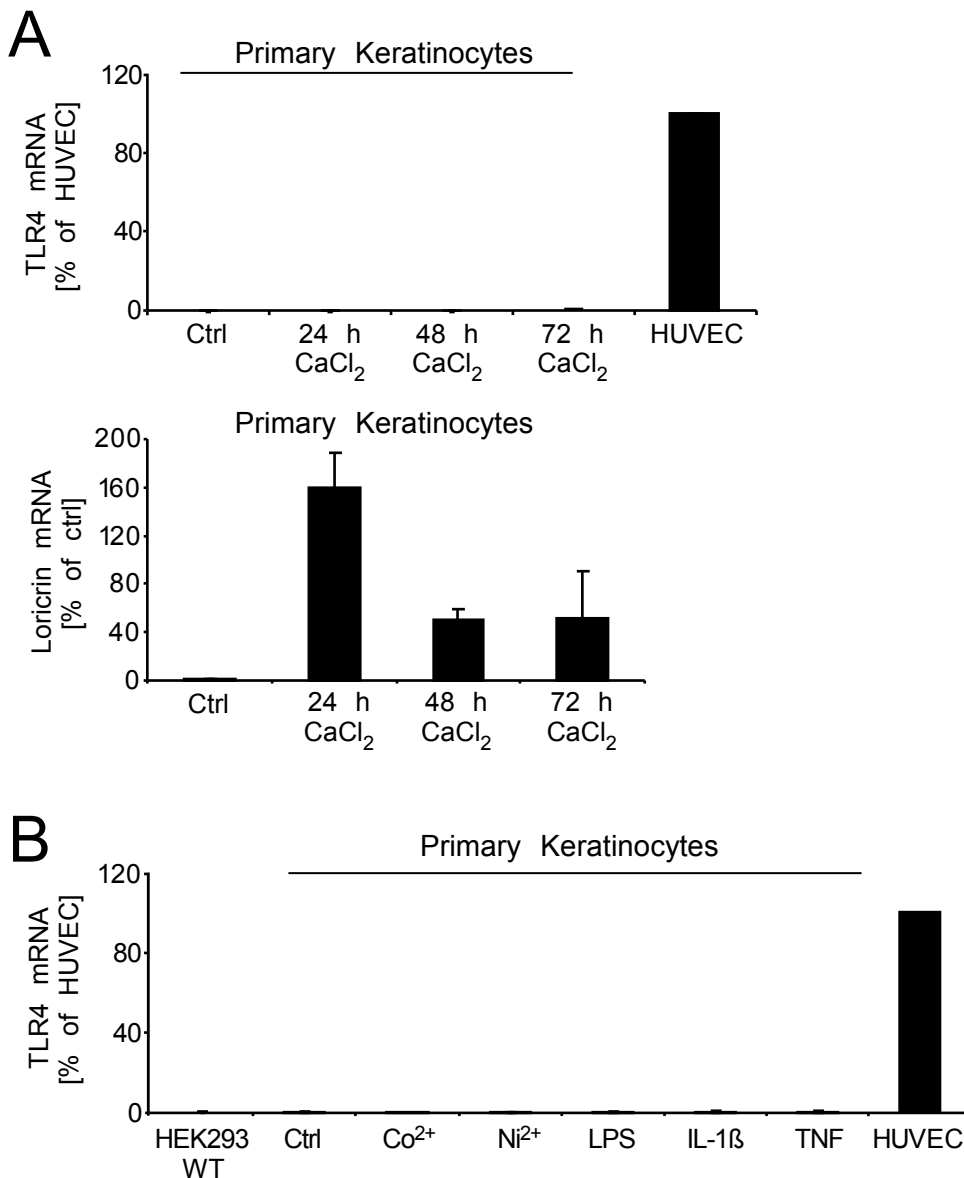
qRT-PCR array. 0.1 - 1 µg spin column-isolated total RNA (RNeasy Mini Kit, Qiagen) was reversely transcribed using RT² First Strand Kit (Qiagen) to obtain cDNA and internal controls. cDNA was then used to simultaneously monitor mRNA expression of 84 different human cytokine and cytokine receptor genes per sample by SYBR-green based qRT-PCR using the RT² Profiler™ PCR Array System (Array PAHS-022, Qiagen). Experiments were performed according to the manufacturer's protocol using the suggested reagents. To allow statistical evaluation, average fold-induction and p-values from four independent experiments were calculated using an analysis template provided by the manufacturer. Data were each normalized to the expression of 4 different housekeeping genes run on the same 96-well plate. Only genes showing a mean regulation of at least 2.5-fold and a p-value <0.05 were considered to be significantly regulated. Genomic DNA contamination potentially masking reliable gene regulation was controlled for using a specific probe run in parallel. Gene-specific primers consistently delivering multiple PCR products or low T_m values as

determined by melting curve analysis were excluded from the analysis to avoid false positives due to unspecific amplification or primer dimers, respectively.

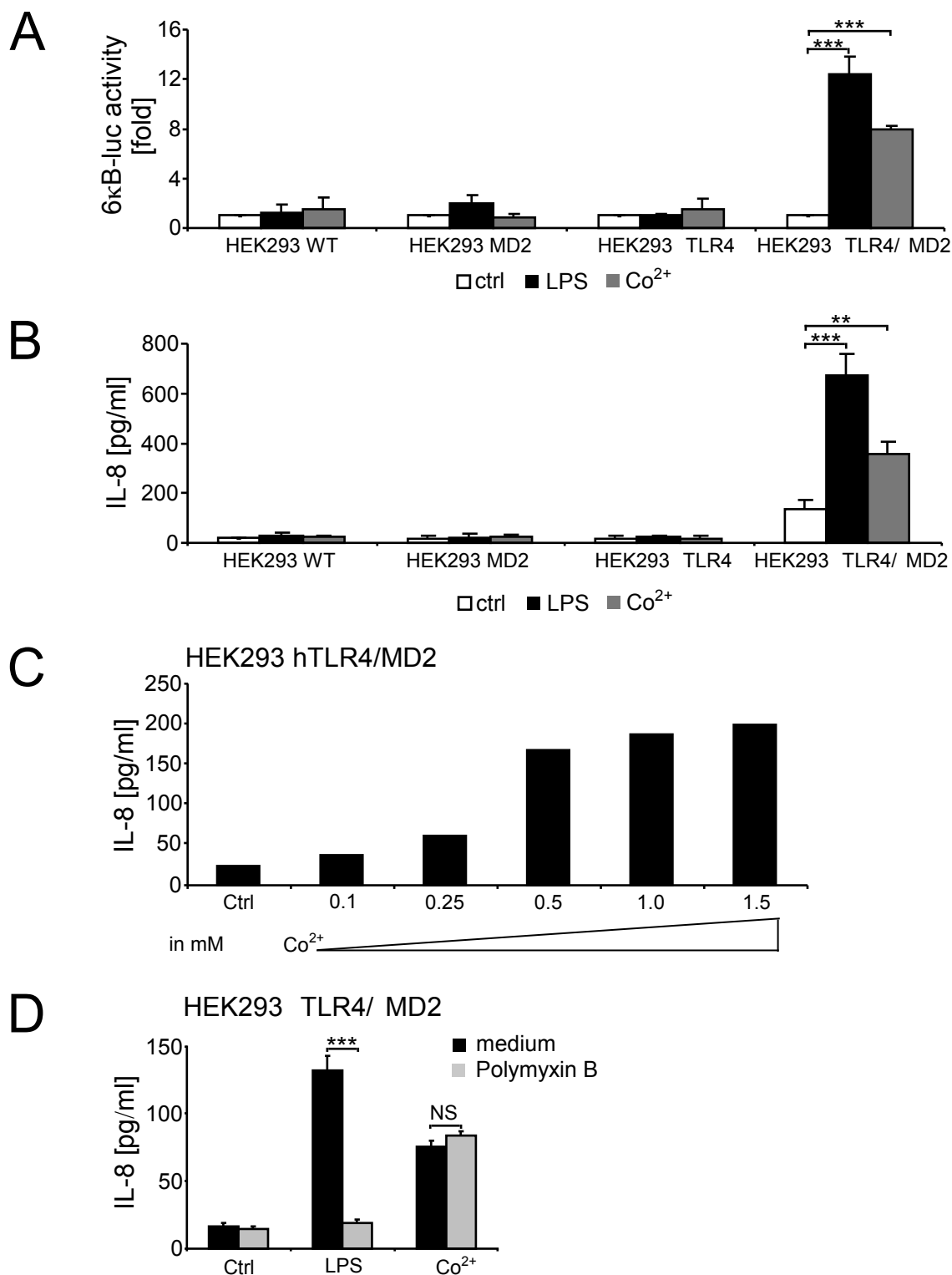
Supplemental References

1. Schmidt M *et al* (2010) Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol* **11**: 814-819

Supplementary Fig S1

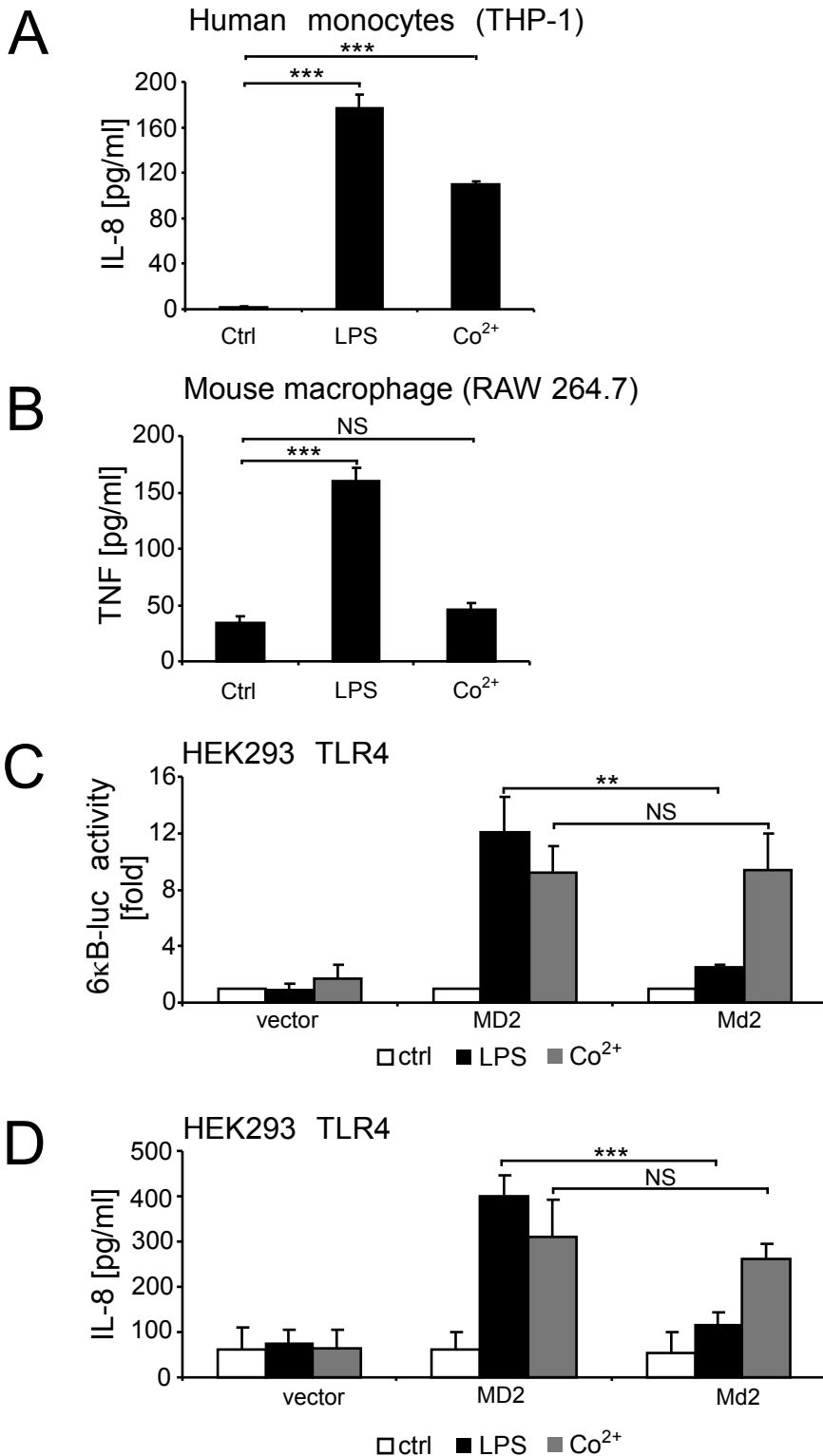


Supplementary Fig S1: Keratinocytes fail to induce TLR4 expression during differentiation or upon proinflammatory activation. qRT-PCR analysis of TLR4 mRNA expression in NHEK (**A**, upper panel and **B**) as compared to TLR4/MD2-negative HEK293 cells or TLR4/MD2-positive HUVEC. RNA was isolated from calcium-differentiated NHEK (1.0 mM CaCl₂ for the indicated times) (**A**), or NHEK treated with the indicated proinflammatory stimuli for 16h (**B**), respectively. After reverse transcription, cDNA was subjected to qRT-PCR analysis using specific Taq-Man probes. Gene expression was each normalized to GAPDH expression. Data are presented as average-fold regulation \pm S.D. as compared to the relative expression in HUVEC (arbitrarily set to 100 %). For confirmation of successful calcium-induced differentiation (**A**, top panel) time-dependent mRNA induction of the epidermal early differentiation marker loricrin (represented as mean fold induction \pm S.D. related to the unstimulated control) was monitored in parallel (**A**, bottom panel). Data in each subfigure represent at least three independent experiments.



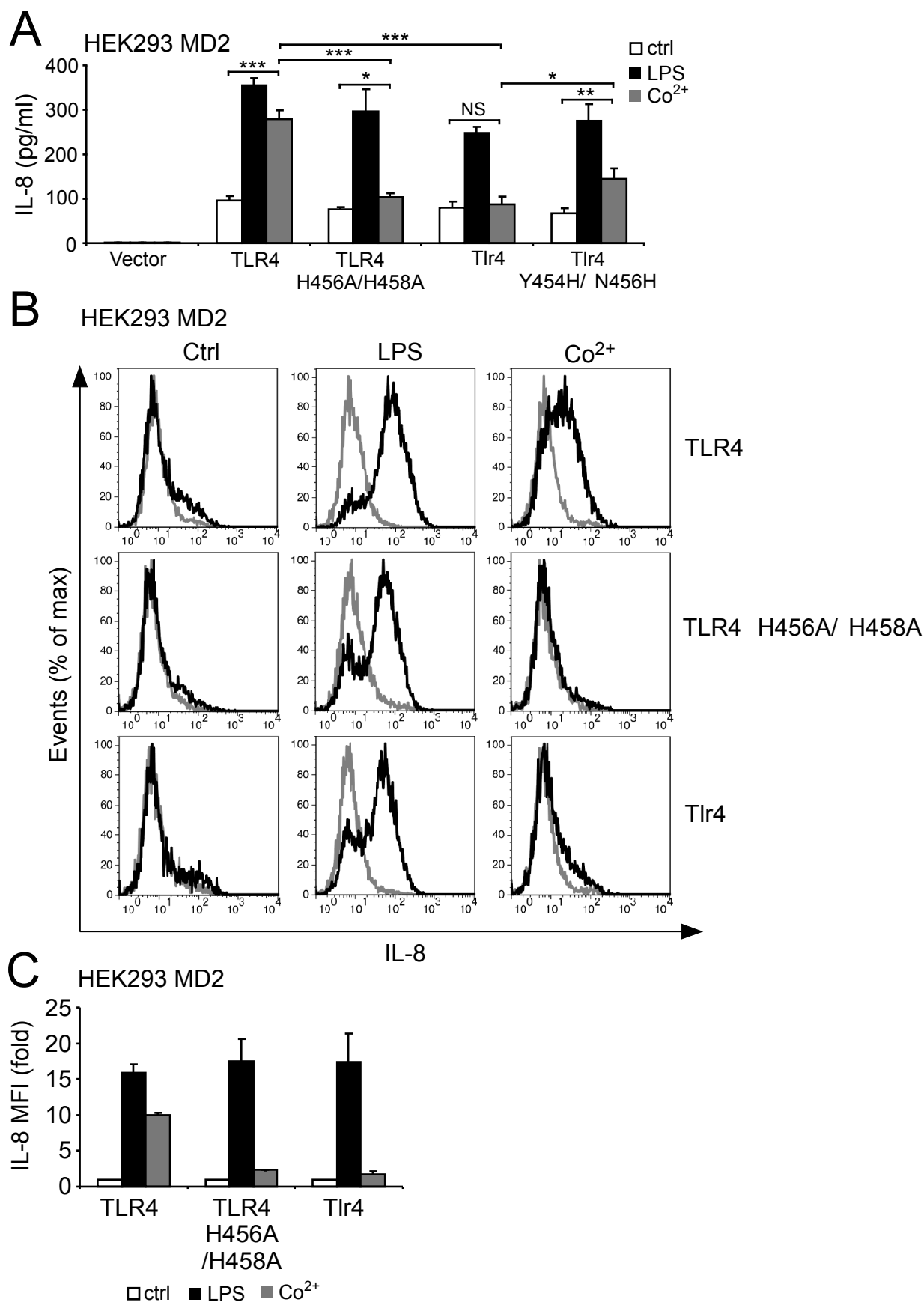
Supplementary Fig S2: Co²⁺-induced proinflammatory gene expression requires TLR4 and MD2. **(A)** Average fold luciferase activation of a 6x κ B luciferase (luc) reporter transfected into HEK293 wildtype cells (HEK293 WT), HEK cells stably expressing human MD2 (HEK293 MD2), TLR4 (HEK293 TLR4) or TLR4 and MD2 (HEK293 TLR4/ MD2), respectively. **(B)** Analysis of Co²⁺-induced IL-8 protein expression in the indicated cell lines as determined by analysis of supernatants by ELISA. Cells were stimulated for 8 h with medium (ctrl), Co²⁺ (1.5 mM) or the TLR4 agonist LPS (*S. minnesota* R595; 1 μ g ml⁻¹) as positive control, respectively. **(C)** Concentration-dependent IL-8 protein expression in supernatants of Co²⁺-stimulated HEK293 TLR4/ MD2 cells as determined by ELISA. **(D)** Quantification of IL-8 in the supernatants of HEK293 hTLR4/ MD2 cells upon 8 h stimulation with medium (Ctrl), 1.5 mM Co²⁺ or 1 μ g ml⁻¹ LPS (*E. coli* 055:B5) in the absence or presence of 50 mg ml⁻¹ polymyxin B sulphate. With exception of **(C)** showing a single experiment, data represent mean fold values **(A)** or average values **(B,D)** \pm S.D. of three independent experiments. ** p < 0.01, *** p < 0.001, NS, not significant, unpaired t-test.

Supplementary Fig S3



Supplementary Fig. S3: Co²⁺-induced gene expression requires sequence motifs present in human TLR4 but not in mouse Tlr4. **(A-B)** ELISA experiments demonstrating species-dependent release of known NFκB-dependent cytokines in macrophages. **(A)** Co²⁺-dependent production of IL-8 in the human monocytic cell line THP-1. **(B)** Failure of Co²⁺ to induce the NFκB target TNF in the mouse macrophage cell line RAW 264.7. Cells were each stimulated for 8 h with medium (ctrl), Co²⁺ (1.5 mM) or LPS (*S. minnesota* R595; 1 μg ml⁻¹) (as functionality control for the Tlr4/Md2 complex). **(C-D)** Co²⁺-induced proinflammatory gene expression requires MD2 but occurs independently of the species origin of MD2. **(C)** Activity analysis of a transfected 6x κB-luc reporter or **(D)** ELISA-based analysis of IL-8 protein production in supernatants of stably TLR4-expressing HEK293 cells (HEK-TLR4) transiently transfected with human MD2, mouse Md2 or empty vector upon stimulation with medium (ctrl), Co²⁺ or LPS, respectively. Data represent averages of three independent experiments ± S.D. ** p < 0.01, *** p < 0.001, NS, not significant, unpaired t-test.

Supplementary Fig S4



Supplementary Fig S4: Non-conserved histidines H456 and H458 in TLR4 mediate Co²⁺-dependent proinflammatory gene expression. Stable HEK293 MD2 cells were transfected with the indicated HA-tagged constructs and exposed to Co²⁺, LPS (1 μ g ml⁻¹) or medium (ctrl) for 8 h. **(A)** IL-8 release upon transfection with the indicated constructs. Data represent averages of three independent experiments \pm S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, not significant, unpaired t-test. **(B)** Flow cytometric staining of intracellular IL-8 protein expressed by the positively transfected cell population. Transfection of the indicated constructs was monitored by HA-immunostaining and gating on the HA-positive population. Representative overlays of IL-8 staining (black lines) and isotype-staining (grey lines) of three independent experiments are shown. **(C)** Flow cytometric quantification of three independent experiments described in **(B)**. Data represent means of isotype-corrected median fluorescence intensities (MFIs) of IL-8-staining calculated for the HA-gated population \pm S.D.